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Temporal Changes in Neurotrophic Factors and Neurite Outgrowth in the Major Pelvic Ganglion Following Cavernous Nerve Injury

Johanna L. Hannan,[1]* Maarten Albersen,[2] Bernard L. Stopak,[1] Xiaopu Liu,[1] Arthur L. Burnett,[1] Ahmet Hoke,[3] and Trinity J. Bivalacqua[1]

[1]The James Buchanan Brady Urological Institute and Department of Urology, Johns Hopkins School of Medicine, Baltimore, Maryland
[2]Laboratory for Experimental Urology, Department of Development and Regeneration, University of Leuven, Leuven, Belgium
[3]Department of Neurology, Johns Hopkins Hospital, Baltimore, Maryland

Despite nerve-sparing radical prostatectomy, nerve damage and erectile dysfunction (ED) prevail, and preventing neurodegeneration is of great importance. Neurotrophic factors and neurite outgrowth were characterized in major pelvic ganglia (MPG) following bilateral cavernous nerve injury (BCNI). Young male Sprague-Dawley rats underwent sham or BCNI surgery, and the intracavernosal pressure to mean arterial pressure ratio was measured 2, 7, 14, 21, 30, and 60 days following injury (n = 8/group). MPG gene expression (qPCR) and Western blot were performed for glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF), neurturin, neurotrophin (NT)−3, NT4, brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor, and activating transcription factor 3 (ATF3). Additional rats were injured, and MPGs were removed 24 hr, 48 hr, 3 days, and 7 days following BCNI (n = 3/group). MPGs were cultured in Matrigel, and neurite outgrowth was measured. Erections were impaired early and improved by 60 days in BCNI rats. GDNF, NGF, BDNF, and ATF3 gene expression was significantly increased and NT3 was decreased in MPGs following BCNI (48 hr to 21 days, $P < 0.05$). GDNF and NGF protein levels were elevated in 48-hr BCNI rats. MPG neurite outgrowth from 24-hr and 48-hr BCNI was higher than sham (658 ± 19 μm, 607 ± 24 μm, 393 ± 23 μm, respectively, $P < 0.05$). Further studies examining the roles of neurotrophic factors in modulating signaling pathways may provide therapeutic avenues for neurogenically mediated ED.

Key words: peripheral nerve injury; erectile dysfunction; neurite outgrowth; activating transcription factor 3

The major pelvic ganglia (MPG) are peripheral mixed ganglia consisting of sympathetic and parasympathetic neurons that innervate the urogenital organs and the lower gastrointestinal tract. The location of the ganglia and cavernous nerve (CN), they are susceptible to neuropraxia or neurectomy during pelvic surgeries, such as radical prostatectomy (RP), which can result in neurogenic complications, including urinary incontinence and erectile dysfunction (ED; Boorjian et al., 2012). To limit this functional consequence of pelvic surgery, so-called nerve-sparing operative techniques have been developed in an attempt to limit nerve damage to neuropraxia, in which spontaneous nerve recovery without surgical nerve realignment is a realistic expectation. To improve erectile function outcomes of RP surgeries further, studies have focused on pharmacological therapies, such as phosphodiesterase 5 inhibitors, to limit end organ damage in the penis, without any success (Montorsi et al., 2014; Pisansky et al., 2014). To develop effective interventions to prevent neurodegeneration and promote neuroregeneration, a better understanding of the intrinsic processes that take place within the injured MPG and CN is required.

The peripheral nervous system has demonstrated the ability to regenerate and sprout new axons after injury, although many factors contribute to the functional outcome. The severity and type of nerve injury, age, or underlying diseases will impact repair and recovery (Scheib and Hoke, 2013). After injury, distal nerves undergo Wallerian degeneration, and a regenerative response is initiated in the neuronal cell bodies (expression of regeneration-associated genes) and in Schwann

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*Correspondence to: Johanna L. Hannan, PhD, The James Buchanan Brady Urological Institute and Department of Urology, Johns Hopkins School of Medicine, 600 N. Wolfe Ave., Marburg 420, Baltimore, MD 21287. E-mail: jhannan2@jhmi.edu

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cells (upregulation of neurotrophins, cytokines, chemokines, and extracellular matrix proteins; Kiryu-Seo and Kiyama, 2011; Scheib and Hoke, 2013). In sciatic nerve injury, neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin (NT)-3 are produced in a temporal fashion to encourage axon growth (Golz et al., 2006). These processes in the MPG are now being studied; however, they are not well defined or have been assessed at only one time point following injury.

To determine the temporal gene expression changes that occur in the MPG following injury, we assessed global gene expression changes in injured MPGs at early (48 hr) and later (14 days) time points. Many significant changes were evident at a transcriptional level involving galanin, GDNF, BDNF, and NGF signaling pathways (Calenda et al., 2012; Weyne et al., 2014). Additionally, exogenous neurotrophic therapies have been used to promote erectile recovery in diabetic or CN injury rat models of ED. These investigations provide support for the use of neurotrophic factor therapy but have not fully addressed the pathogenesis of CN dysfunction that occurs in the MPG to promote neuritogenesis and regeneration.

In addition to the CN injury rat model of ED, the MPG can be excised and grown on Matrigel to access its ability to sprout and grow neurites. This is an experimental tool that can be used to access the ability of different compounds to inhibit or promote MPG neurite growth. Neurite outgrowth in explanted MPGs grown in culture is dependent on neurotrophic factor stimulus (Lin et al., 2003; Bella et al., 2006; Lin et al., 2006). In general, neurotrophic factors alone or in combination have been shown to enhance MPG neurite outgrowth in young and old MPGs (Lin et al., 2010). MPG Matrigel culture following CN injury has yet to be assessed. In dorsal root ganglia (DRG) culture, in vivo sciatic nerve injury leads to twice the length of DRG neurite outgrowth compared with uninjured DRG (Aguis et al., 1998). Injury can prime the nerves for outgrowth by upregulating intrinsic growth factors to allow for repair and regeneration. We sought to determine whether the increased gene expression of neurotrophic factors seen in vivo following CN injury could impact the neurite outgrowth of the MPG in vitro.

This study examines the time course of intrinsic neurotrophic factors from the GDNF and NGF families in the MPG following a moderate CN crush injury. The degree of injury was assessed by measuring a marker of nerve injury, activating transcription factor 3 (ATF3), and recording erectile function. Additionally, we assessed the time course of neurite outgrowth in cultured control and CN-injured MPGs to determine whether the intrinsic rise in neurotrophic factors could increase neurite outgrowth in MPGs.

MATERIALS AND METHODS

Animals and Experimental Design

Male Sprague Dawley rats (n = 119; Charles River, Wilmington, MA) weighing 250–300 g were used in this study. Rats had ad libitum access to standard rat chow and water. Rats underwent sham (n = 16), or bilateral CN injury (BCNI) surgery, and tissues were harvested 48 hr (BCNI 48-hr rats n = 16), 7 days (BCNI 7-day rats n = 16), 14 days (BCNI 14-day rats n = 16), 21 days (BCNI 21-day rats n = 8), 30 days (BCNI 30-day rats n = 16), or 60 days (BCNI 60-day rats n = 16) after injury to be used for molecular studies. Additional rats underwent sham and BCNI surgeries, and MPGs were collected 24 hr, 48 hr, 72 hr, or 7 days after injury and cultured in Matrigel (n = 3/group). All experiments were conducted in accordance with the Johns Hopkins University School of Medicine guidelines for animal care and use, the NIH *Guide for the care and use of laboratory animals*, and the Society of Neuroscience guidelines for the use of animals in neuroscience and behavioral research.

BCNI

With animals under isoflurane anesthesia, the prostate was exposed via a midline laparotomy, and the MPG and CN were identified bilaterally. In the BCNI groups, both CNs were injured by crushing with forceps three times for 15 sec each 2–3 mm distal to the MPG (Hannan et al., 2013, 2014). Adequate crush was confirmed by an observable change in nerve color to gray, with the neurolemma remaining intact. In sham animals, the CN was identified, and the abdomen was closed. BCNI surgeries were all performed by the same surgeon.

Measurement of Erectile Responses

With animals under anesthesia, the right CN was identified. The right cru was cannulated with a 25-G needle connected to a pressure transducer to measure intracavernous pressure (ICP). The right carotid artery was cannulated for continuous measurement of mean arterial pressure (MAP). The CN distal to the crush injury was stimulated with a square pulse stimulator (Grass Instruments, Quincy, MA) at a frequency of 20 Hz, duration of 0.5 msec, and pulse width of 30 sec at increasing voltages (2, 4, 6, and 8 V) for 1 min, with 3–5 min between stimulations. The ratio between maximal ICP and MAP obtained at the peak of erectile response was calculated to normalize for variations in systemic blood pressure. Total ICP was measured as the area under the curve (AUC) during the time of stimulation.

Major Pelvic Ganglia Culture and Neurite Outgrowth Assessment

After sham or BCNI surgeries, MPGs were carefully dissected at 24 hr, 48 hr, 72 hr, or 7 days after injury to be cultured in Matrigel (n = 3/group). Whole MPGs were carefully separated from the prostatic capsule, excised, and kept on ice in serum-free media (RPMI 1640 with 1% penicillin–streptomycin; Gibco, Grand Island, NY) until they were embedded. Reduced growth factor Matrigel was diluted with media, and 200 μl was placed on the bottom of a 24-well plate. After polymerization, MPGs were placed in the center of the well, covered with 300 μl diluted Matrigel, and allowed to harden for 30 min at 37°C. MPGs were covered with 1 ml media with

vascular endothelial growth factor (VEGF; 25 μg/ml; R&D Systems, Minneapolis, MN), which was changed every 24 hr and maintained at 37°C in a humidified atmosphere with 5% CO_2. Photographs of neurite growth at 24, 48, and 72 hr were captured with a Nikon TE200 inverted microscope attached to a CCD camera, and digital images were analyzed in Elements software (Nikon Instruments, Melville, NY). In each area of growth from the MPG, the five longest neurites were measured. The averages of these measurements defined the neurite length for groups at each time point (20–25 neurites measured per MPG).

Quantitative PCR

Real-time quantitative PCR (qPCR) was used to determine relative expression of neurotrophic factors in MPGs from sham, 48-hr, 7-day, 14-day, 21-day, 30-day, and 60-day BCNI rats. Frozen MPGs were homogenized, and total RNA was purified with the RNeasy system (Qiagen, Hilden, Germany), quantified, and then reverse transcribed with Ready-To-Go You-Prime first-strand beads (GE Healthcare, Pittsburgh, PA). Real-time qPCR was performed with the StepOnePlus system (Applied Biosystems, Foster City, CA). TaqMan gene expression assays for GDNF (Rn00569510), VEGF (Rn01511601), BDNF (Rn02531967), neurturin (NRTN; Rn01527513), NGF (Rn01533872), NT3 (Rn00579280), NT4 (Rn00566076), ATF3 (Rn00563784), and hypoxanthine phosphoribosyltransferase 1 (HPRT1; Rn01527840) were used. HPRT1 was unchanged between groups and served as an endogenous control in which all values were normalized to HPRT1 transcript levels (Applied Biosystems). All experiments were performed on eight separate whole MPG cellular fractions from each group with triplicate technical replicate PCRs per sample.

Western Blot Analysis

Whole, paired MPGs (n = 8/group) were excised from sham, 48-hr, 7-day, 14-day, 30-day, and 60-day BCNI rats and homogenized in cell lysis buffer (Cell Signaling Technology, Beverly, MA). Cellular fractions from homogenized MPGs were isolated for GDNF and NGF Western blot analysis. Protein amounts were determined with the BCA kit (Pierce, Rockford, IL), and equal amounts of protein (30 μg) were loaded onto 4–20% Tris-HCl gel (Bio-Rad, Hercules, CA). After their separation by SDS-PAGE, the proteins were transferred to polyvinylidene fluoride membranes and incubated with primary antibodies (GDNF 1:200, NGF 1:200, and glyceraldehyde-3-phosphate dehydrogenase [GAPDH] 1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The membranes were incubated with a horseradish peroxidase-linked secondary antibody and visualized with an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ). The densitometry results were normalized by GAPDH expression, which was unchanged between groups. The intensities of the resulting bands were quantified in ImageJ (NIH, Bethesda, MD).

Statistical Analysis

Data are expressed as mean ± SEM. Differences between multiple groups were compared by one-way ANOVA, followed by Tukey's multiple-comparisons test (GraphPad Prism 5; GraphPad Software, San Diego, CA). ATF3, GDNF, and NGF expression and ICP/MAP values from sham and all BCNI time points were correlated by calculating Spearman's rank correlation coefficient. $P < 0.05$ was considered statistically significant.

RESULTS

Erectile Responses

Erectile function was significantly lower at all time points in a voltage-dependent manner following BCNI (Fig. 1). At early time points after injury, a marked decrease was demonstrated in both ICP/MAP and AUC, which was lowest at 14 days. Erectile responses began to increase gradually from 30 to 60 days following BCNI, and at a stronger electrostimulation (6 V) 60-day injured rats had significantly increased ICP/MAP compared with BCNI 7–30-day rats ($P < 0.05$). Although erections were improved at 60 days following BCNI, they remained significantly lower than those in sham animals ($P < 0.05$). These results are further illustrated in the representative ICP and MAP tracings for each group (Fig. 1C).

Neurotrophic Factor Gene and Protein Expression

To confirm that the decrease in erectile response was due to CN injury, we measured the gene expression of a marker of neuronal injury, ATF3, in the MPG. ATF3 was increased at all time points following BCNI; however, it reached significance only at 48 hr, 14 days, and 21 days postinjury ($P < 0.05$; Fig. 2A).

The gene expression of several neurotrophic factors was assessed in the MPG following injury. BDNF gene expression was significantly increased after 7 days ($P < 0.05$) and had returned to sham values by 14 days after BCNI (Fig. 2B). VEGF, a known vasculogenic growth factor with neurotrophic properties, and NRTN, a neurotrophic factor related to the GDNF family, were not different following BCNI (Fig. 2C,D). NT3 and NT4 are members of the NGF family of neurotrophic factors. The gene expression of NT3 was significantly decreased at early time points (48 hr and 7 days), was temporarily increased at 14 and 21 days, and was lowered again at 30 and 60 days following BCNI ($P < 0.05$; Fig. 2E). NT4 remained unchanged at all time points following nerve injury (Fig. 2F).

The most pronounced change in neurotrophic factor gene expression following BCNI was GDNF. GDNF mRNA increased ~20-fold 48 hr after injury and remained elevated five- to sevenfold up to 21 days post-BCNI ($P < 0.05$; Fig. 3A). At later time points (30–60 days), GDNF gene expression was not different from sham values. NGF gene expression was also significantly elevated 48 hr, 14 days, and 21 days after crush injury ($P < 0.05$) and returned to sham baseline at 30 and 60 days post-BCNI (Fig. 3B). Western blots were performed for these two neurotrophic factors because they had the most pronounced changes in gene expression. GDNF protein amounts were significantly lower 48 hr following

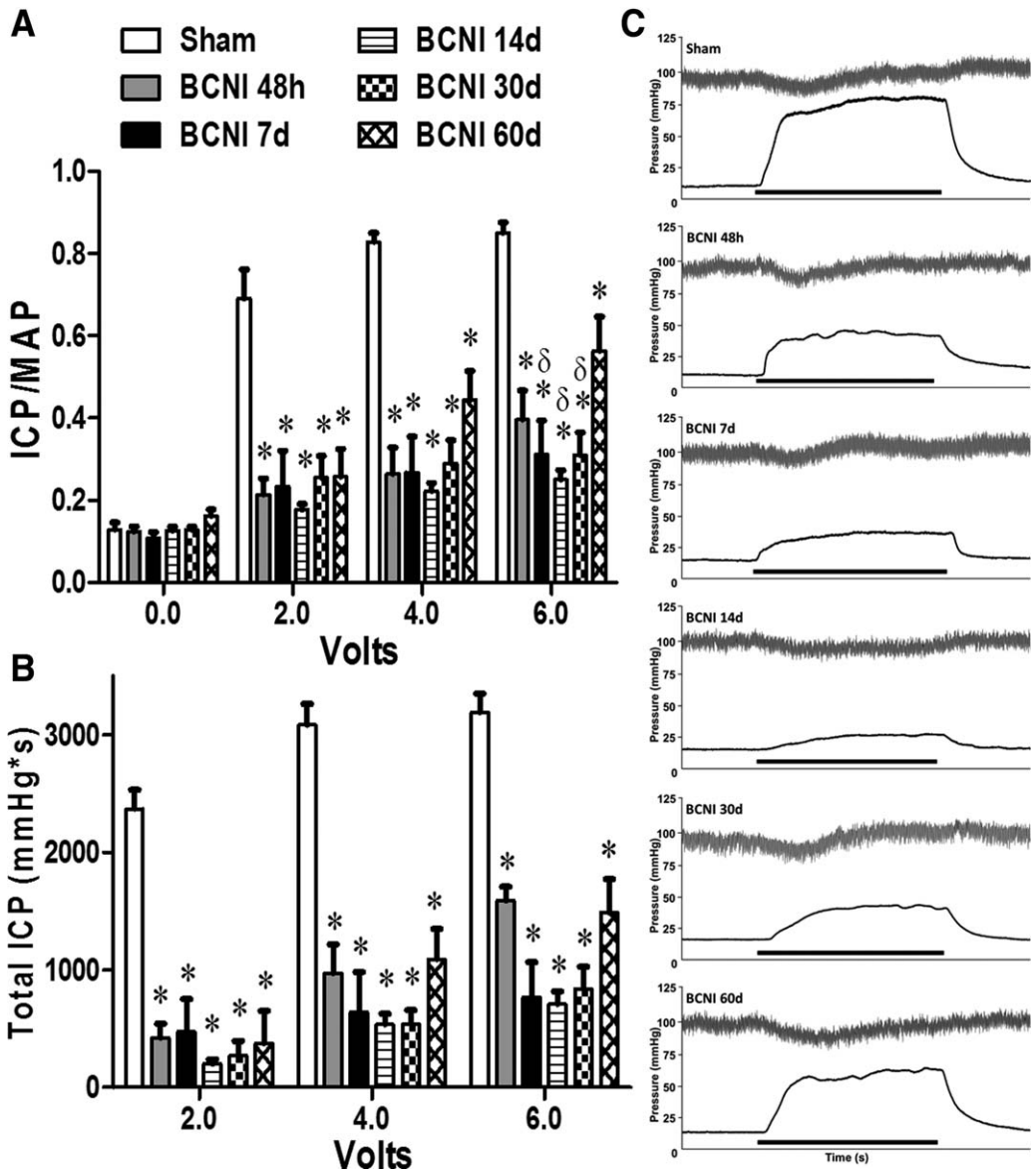

Fig. 1. In vivo erectile responses. In vivo erectile responses were assessed in sham and BCNI rats 48 hr and 7, 14, 30, and 60 days (n = 8/group) after BCNI via electrostimulation of the cavernous nerve. Bar graphs show voltage-dependent erectile responses, measured by the ICP to MAP ratio (**A**) and total ICP (area under the erectile curve (**B**) after CNS for 1 min in all groups. Representative tracing of ICP and MAP responses for each group are shown at 6-V stimulation for 1 min, indicated by the solid bar along the x-axis (**C**). Data are mean ± SEM. $^{\star}P < 0.05$ compared with sham rats; $^{\delta}P < 0.05$ vs. BCNI 60-day rats.

injury ($P < 0.05$) and at 7 days had returned to, and were maintained at, sham levels (Fig. 3C). Similarly to GDNF, NGF protein levels were significantly lower 48 hr following injury and were normalized by 14 days post-BCNI (Fig. 3D).

Correlation of Erectile Function and Neurotrophic Factors

Erectile response data (ICP/MAP) recorded after 6-V electrical stimulation were correlated with the corresponding mRNA ATF3, GDNF, or NGF values. Spearman's rank correlation coefficients between ATF3, GDNF, and NGF gene expression and erectile responses were −0.8264, −0.605, and −0.659, respectively ($P < 0.0001$). ATF3 gene expression levels were significantly increased when erectile responses were low, and ATF3 mRNA levels were much lower when erections had returned to sham levels (Fig. 4B). This strong negative correlation demonstrates that GDNF and NGF gene expression values are high when erectile responses are impaired.

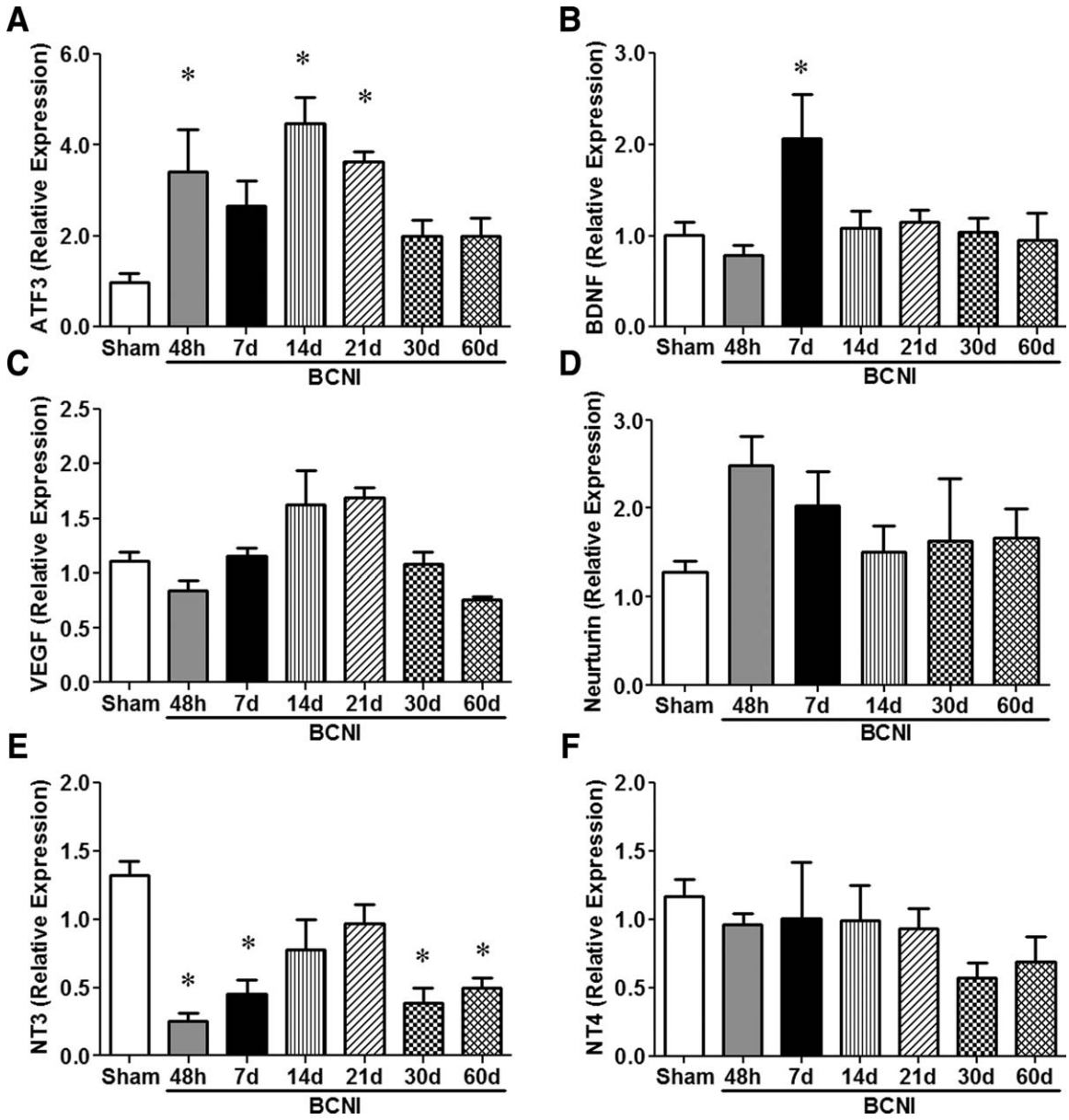

Fig. 2. Gene expression of neurotrophic factors. Bar graphs show MPG gene expression of ATF3 (**A**), BDNF (**B**), VEGF (**C**), NRTN (**D**), NT3 (**E**), and NT4 (**F**) from sham-operated rats and various time points from rats following BCNI (n = 8/group). The gene expression levels were normalized to the gene expression level for GAPDH. Data are mean ±SEM. $^{\star}P < 0.05$ compared with sham rats.

Neurite Outgrowth

The time course of neurite outgrowth was assessed in MPGs cultured at 24 hr, 48 hr, 72 hr, and 7 days after BCNI. Neurite outgrowth was measured after the MPGs were incubated for 24, 48, and 72 hr. In all groups, neurite outgrowth increased with incubation time (Fig. 5). A significant increase in neurite growth was seen in MPGs among rats injured 24 and 48 hr prior to culture compared with sham MPGs ($P < 0.05$). BCNI 72-hr MPGs grew less than sham MPGs after 24 hr of incubation but were no different at 48 or 72 hr of incubation. MPGs that had been injured 7 days prior to culture grew similarly to sham MPGs after 24 and 48 hr; however, at 72 hr of incubation, they showed significantly increased growth vs. sham MPGs ($P < 0.05$).

DISCUSSION

This study demonstrates the temporal changes in neurotrophic factors in the injured MPG at early time points when erections are severely diminished, reaching their

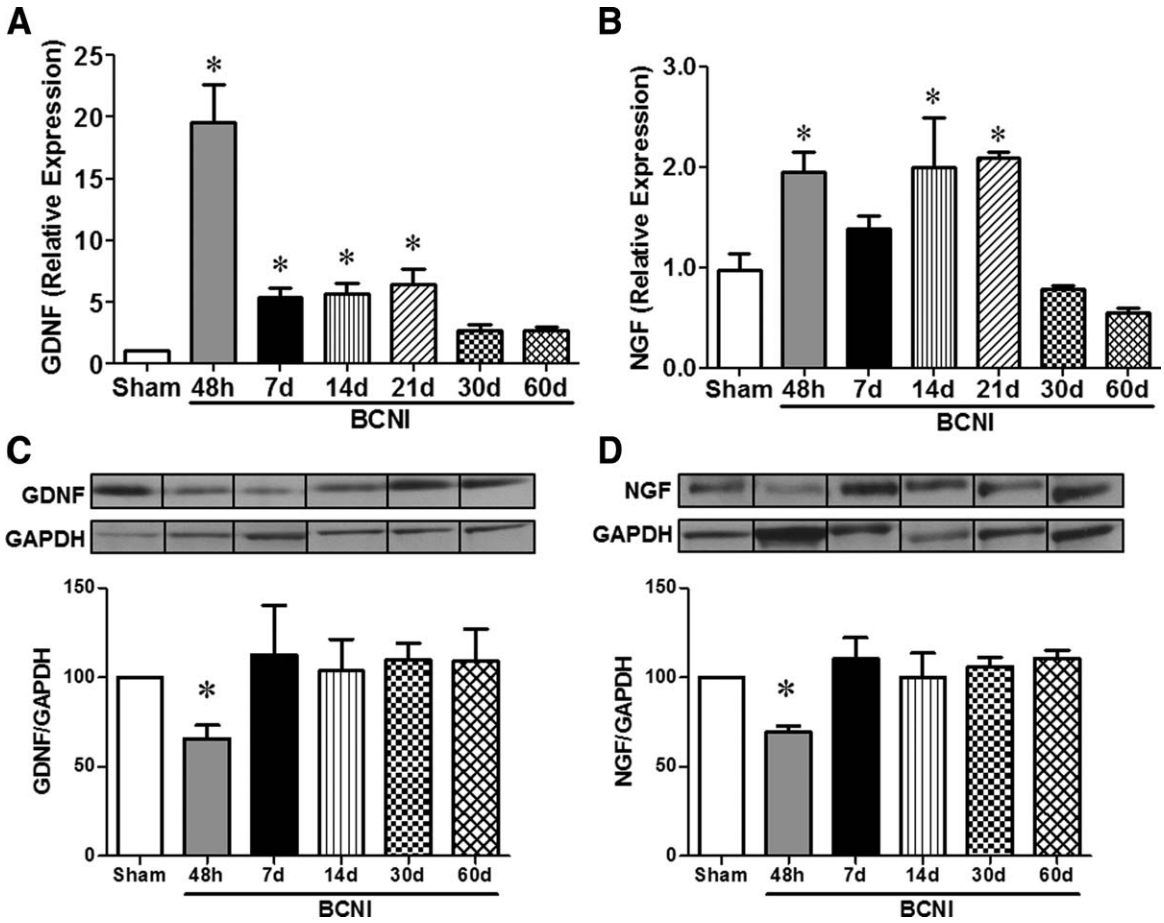

Fig. 3. Gene and protein expression of GDNF and NGF. Bar graphs show MPG gene expression of GDNF (**A**) and NGF (**B**) from sham-operated rats and various time points from rats following BCNI (n = 8/group). GDNF and NGF gene expressions were normalized to relative expression of GAPDH. Representative Western blots and bar graphs for GDNF (**C**) and NGF (**D**) protein expression in MPGs from all groups. Data are normalized to GAPDH protein expression. Data are mean ± SEM (n = 8/group). $^{\star}P < 0.05$ compared with sham rats.

lowest values after 14 days and beginning to improve by 30–60 days. The gene expressions of ATF3, GDNF, NGF, and BDNF were temporally increased and NT3 was decreased at times when erectile function was impaired. GDNF and NGF had the greatest increase in MPG gene expression following CN injury. At later time points, when erectile function was improved, all neurotrophic factors had returned to sham values. Neurite outgrowth from cultured MPGs was potentiated when the MPG was harvested 24–48 hr after injury, indicating a possible neuroregenerative effect of the observed early ATF3 and neurotrophin upregulation.

We recently demonstrated a time course of spontaneous recovery of erectile function following BCNI with ICPs stimulated at 8 V (Weyne et al., 2014). This study uses the same time course following injury but demonstrates the voltage responses from 2 to 6 V. Results similar to ours were seen in BCNI mice that had a slight increase in erectile function at 4 weeks and significant improvements in erections at 8 and 12 weeks following injury (Jin et al., 2010). Mice with CN transection demonstrated no recovery of erections from 3 days to 12 weeks postinjury. These data demonstrate the importance of evaluating early and late time points to ensure that improvements in erectile function following therapeutic interventions are not due to intrinsic recovery of neuronal function.

ATF3 is an early gene upregulated in response to injury (Tsujino et al., 2000) that enhances peripheral nerve regeneration by promoting the growth of injured neurons (Seijffers et al., 2007) and increases neuronal survival (Herdegen et al., 1997). After axotomy of the sciatic nerve, ATF3 mRNA expression was significantly increased within 12 hr of injury, was decreased slightly after 6 weeks, and remained elevated in the DRG for 70 days (Tsujino et al., 2000). Explant cultured MPGs expressed increased ATF3 mRNA 8 hr after being

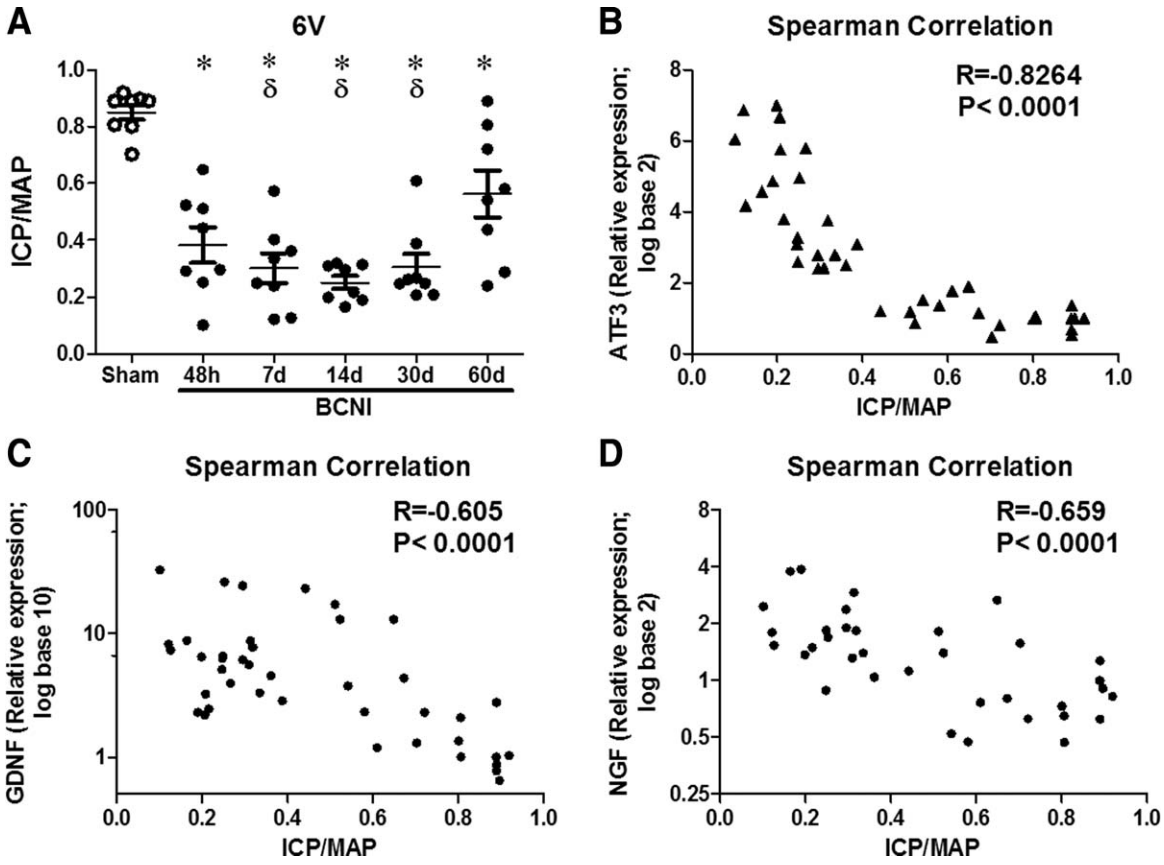

Fig. 4. Relationship between ATF3, GDNF, or NGF and erectile responses. Dot plot shows the erectile responses, measured by the ICP/MAP ratio after cavernous nerve stimulation at 6 V for 1 min in sham, 48-hr, and 7-day, 14-day, 30-day, and 60-day BCNI rats (**A**). Data are mean ± SEM (n = 8/group). Scatter diagrams show the relationship between MPG ATF3 (**B**), GDNF (**C**), or NGF (**D**) gene expression level and ICP/MAP to cavernous nerve electrostimulation at 6 V from sham and all injured time points. R, Spearman correlation coefficient. $^{\star}P < 0.05$ ICP/MAP response compared with sham rats; $^{\delta}P < 0.05$ ICP/MAP response vs. BCNI 60-day rats.

cultured, and transcript levels were elevated 84-fold after 3 days in culture (Girard et al., 2010). Our results are similar to the crush-injured DRG results because there was a significant increase in ATF3 gene expression at 48 hr, followed by a decrease at 30 and 60 days. There was also a very strong negative correlation between ATF3 transcript levels and erectile function. Further studies are warranted to determine its specific role in CN regeneration and confirm whether it can serve as a marker of CN injury in the MPG.

GDNF, which promotes the survival of central and peripheral neurons, was determined to have the greatest increase in MPG gene expression following injury (Treanor et al., 1996). The expression of GDNF inversely correlated with erectile function. After CN transection, there is a decrease in GDNF mRNA expression in the penis, and GDNF protein undergoes retrograde transport from the penis to the MPG (Laurikainen et al., 2000). The current study provides evidence that GDNF is also produced locally in the MPG to promote CN survival in an autocrine fashion. Increasing the expression of GDNF following CN injury via herpes simplex viral (HSV) vector delivery targeted to the MPG has been successful at recovering erectile function (Kato et al., 2007).

Another member of the GDNF family that promotes neuron growth and survival is NRTN. Although we did not see any significant changes in the gene expression of NRTN in the injured MPG, many others have reported its benefits for the treatment of neurogenic ED. Extended release NRTN placed directly onto the MPG following nerve crush leads to moderate improvement in ICP responses 5 weeks after injury (Bella et al., 2007a). NRTN HSV gene transfer also only mildly increased erectile responses in CN injured rats (Kato et al., 2009). Although NRTN appears to have had a modest beneficial effect in both rat studies, erections were not recovered to sham levels. The current data support these findings in that NRTN was not activated in response to injury.

NGF was one of the first growth factors to be described and was shown to increase growth of MPG cultured neurons (Tuttle and Steers, 1992; Tuttle et al., 1994). NGF was assessed as an in vivo treatment alone or

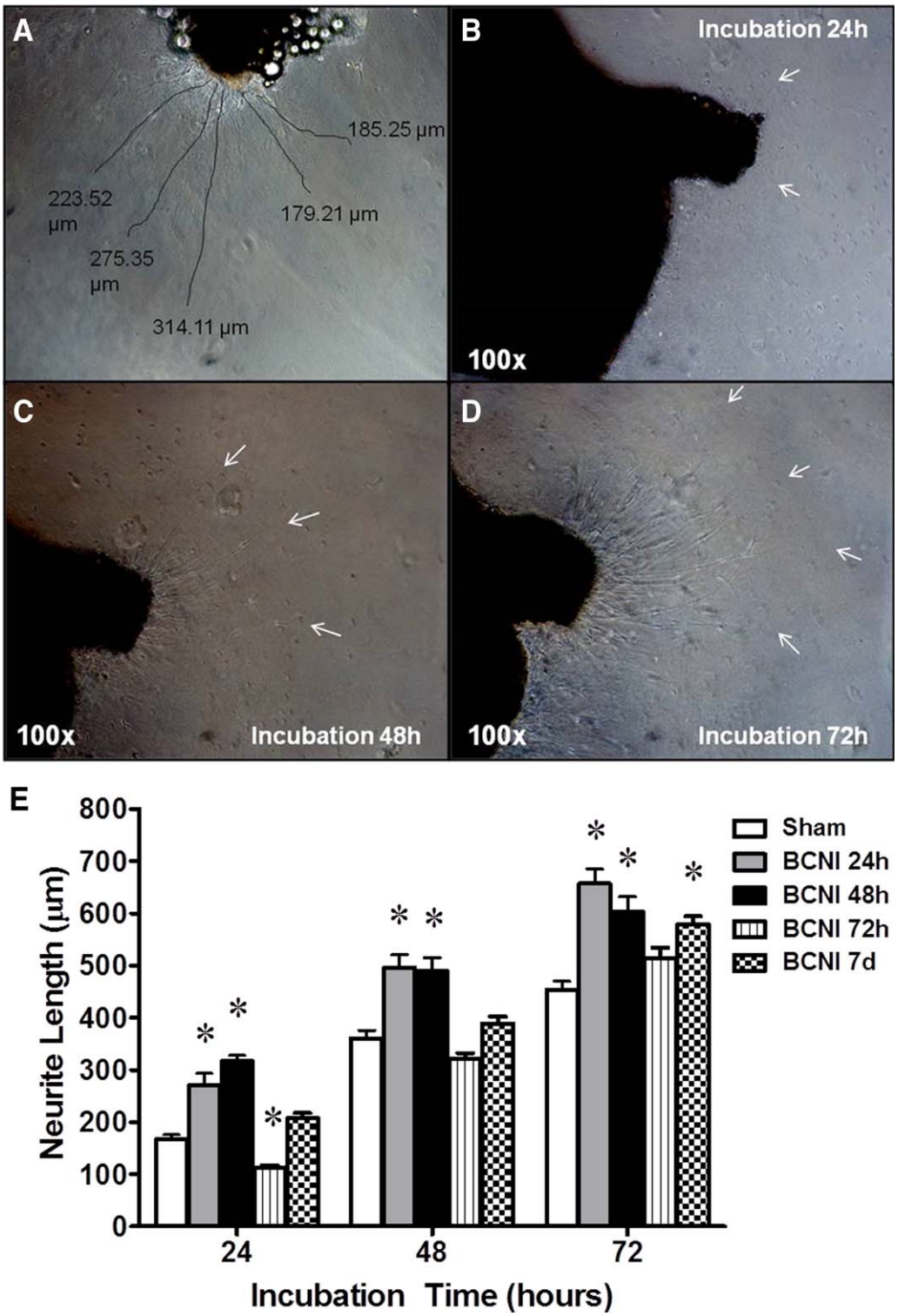

Fig. 5. Time course of MPG neurite outgrowth following injury. Representative images of neurites sprouting from MPG from sham animals in the presence of VEGF (**B–D**) cultured in vitro and the methods of analysis (**A**). Images were all taken at ×100; arrows indicate length of neurite. **E:** Bar graph shows neurite length of MPGs from sham, 24-, 48-, and 72-hr and 7-day BCNI rats after 24, 48, and 72 hr in culture. Data are mean ± SEM (n = 3/group). $\star P < 0.05$ compared with sham rats at corresponding time points. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in combination with nerve grafts following CN ablation in rats (Burger et al., 1991). Treatment with NGF alone minimally increased erectile function, whereas in combination with nerve grafts erections were restored (Burger et al., 1991). The current findings show a correlation between lower MPG NGF gene expression when neurogenic erectile responses have improved. Additionally, NGF was located primarily in the neuronal cell bodies and fibers in the MPG. In peripheral nerves, NGF is transported in a retrograde fashion from the target organ and promotes survival (Zweidel et al., 2005). These data suggest that NGF is an important growth factor in peripheral CN regeneration; however, on its own, it may not be a potent enough growth factor to recover neuronal function fully.

BDNF is an important neurotrophin that supports survival of neurons by contributing to the growth, differentiation, and maintenance of neurons. The protein expression of BDNF in the MPG following crush or axotomy has been characterized at early time points (Bella et al., 2007b; Bond et al., 2013). After axotomy, BDNF protein levels and gene expression levels in the MPG were increased after 1 and 5 days (Bella et al., 2007b); however, after CN crush, BDNF was increased after 1 day, had decreased to below sham levels by 4 days, and was normalized after 7 days (Bond et al., 2013). The current results indicate that BDNF gene expression is significantly increased at 7 days and normalized at 14 days. In the rat, overexpression of BDNF delivered intracavernosally via an adeno-associated virus improved erectile responses 4 and 8 weeks following CN injury (Barkicioglu et al., 2001). It is acknowledged that BDNF protein levels were not assessed in the current study, and further investigation is warranted to determine the role of BDNF in CN regeneration.

NT3, which is responsible for survival and growth of new neurons, was the only neurotrophic factor assessed that demonstrated significantly decreased gene expression in the injured MPG. These results differ from results for MPG gene expression of NT3 following CN axotomy, in which NT3 was unchanged at 1 and 5 days (Bella et al., 2007b). We did not see any changes in NT4 or VEGF gene expression following BCNI. It is noteworthy that most studies demonstrating success at increasing MPG neurite growth in vitro or improving erectile function have used these nerve growth factors in combination with each other or BDNF (Hsieh et al., 2003; Lin et al., 2003). Combination treatments in which BDNF and VEGF are overexpressed have been successful at recovering erectile function following CN injury by freezing and/or crush (Hsieh, 2003; Chen, 2005). It is possible that preservation of nerve function and prevention of neurodegeneration will require a cocktail of nerve growth factors. Furthermore, based on our gene expression data, the timing of administration of different growth factors might also be critical for treatment.

Although we have demonstrated the time course of gene expression of nerve growth factors following CN injury, there is a disconnection between the transcriptional regulation and the protein expression for GDNF and NGF. One week after CN injury, GDNF and NGF protein expression had returned to sham levels; however, gene expression remained elevated up to 21 days. It is possible that, although protein levels have normalized, there are changes in specific neuron populations that might account for the increased gene expression. After CN axotomy, there was a greater decrease in MPG immunofluorescent staining of GDNF family receptors in parasympathetic vs. sympathetic neurons (Palma and Keast, 2006). Future studies will serve to assess costaining of nerve growth factors and markers of sympathetic, parasympathetic, and neuronal nitric oxide synthase-positive neurons in sham and injured MPGs.

Neurite outgrowth from MPG neurons can be stimulated by neurotrophic factors. To date, most studies have looked at uninjured explanted MPGs. Prior injury to a peripheral nerve is termed "conditioning" in DRGs and has been shown to increase neurite growth (Smith and Skene, 1997). We demonstrated that MPGs from animals injured 24 or 48 hr prior to explant culture had the greatest neurite outgrowth compared with intact MPGs. Additionally, MPGs from later injury time points (3 or 7 days) did not demonstrate as much growth as the early injured MPGs (24 or 48 hr). These results are in agreement with the neurotrophic factor gene expression data, which was highest 48 hr after BCNI and had decreased by 7 days after BCNI. Additional studies are required to determine whether there are changes in the sympathetic, parasympathetic, and nitrergic types of neurites that grow in intact vs. injured MPGs and to determine whether there is a difference in the response to growth factors.

One of the limitations to the current study is that we do not have protein expression results for all of the nerve growth factors that were assessed. Furthermore, it would be of interest in future studies to assess the distal nerve stump and the target organ to compare the differences in growth factors to those in the MPG. Future studies will also be required to assess the change in growth factor distribution in the MPGs from injured rats by immunofluorescent staining.

CONCLUSIONS

Prevention of ED in men undergoing a radical prostatectomy will become possible only when the molecular targets leading to neurodegeneration and neurogenesis following CN injury are fully understood. This study demonstrates that neurotrophic factors, such as ATF3, GDNF, and NGF, are significantly elevated following CN injury. Increased neurite outgrowth is evident in 24- and 48-hr injured MPGs, which corresponds to the increased neurotrophic gene expression levels demonstrated after BCNI in vivo. The prevention of neurodegeneration and promotion of survival of axons at an early stage after nerve injury are critical. A cocktail of neurotrophic factors administered at critical time points may be required to prevent neurodegeneration. Additional studies examining the roles of neurotrophic factors in modulating neuroregenerative signaling pathways may provide future therapeutic avenues for neurogenic-mediated ED.

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